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Effect of number of motile, frozen-thawed boar sperm and number of fixed-time inseminations on fertility in estrous-synchronized gilts☆

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ABSTRACT

There are advantages for use of frozen-thawed boar sperm (FTS) as a tool for preservation and transfer of valuable genetic material, despite its practical limitations. It was hypothesized that increasing the number of motile FTS and number of fixed-time artificial inseminations (AI) would improve pregnancy rate and litter size. Semen from six boars was frozen in 0.5 mL straws at 500×10^6 cells/mL. Gilts ~170 days of age, were induced into estrus with PG600® and synchronized using MATRIX™ (synthetic progestagen). Following last feeding of MATRIX (LFM), gilts were checked twice daily for estrus. At onset of estrus, gilts were randomly assigned in a 3×2 factorial treatment design to receive 1×10^9 motile FTS ($n = 19$), 2×10^9 motile FTS ($n = 19$), 4×10^9 motile FTS ($n = 19$) in a single AI at 32 h after onset of estrus, or 1×10^9 motile FTS ($n = 18$), 2×10^9 motile FTS ($n = 17$), or 4×10^9 motile FTS ($n = 19$) in each of the two AI at 24 and 32 h following onset of estrus. Ultrasonography was performed at 12 h intervals after estrus to estimate time of ovulation. Reproductive tracts were collected 28–34 days following AI. Estrus occurred at 139 ± 2 h (mean ± SE) after LFM and ovulation at 33 ± 1 h following onset of estrus. Dose and number of inseminations did not interact or individually influence pregnancy rate at slaughter ($73 \pm 4.2\%$) or numbers of normal fetuses (10.8 ± 0.5). However, number of fetuses tended ($P = 0.14$) to increase with double AI but not with dose. Boar did not affect pregnancy rate but did affect number of normal fetuses and embryonic survival ($P < 0.01$). Longer intervals from insemination to ovulation reduced pregnancy rate ($P < 0.05$), number of normal fetuses ($P < 0.001$), and embryonic survival ($P < 0.01$). Ovarian abnormalities at slaughter were associated with reduced pregnancy rate ($P < 0.001$). The results of this experiment indicate that a double insemination using 2×10^9 motile sperm would produce the greatest number of piglets with fewest numbers of frozen sperm used, while double AI with 1×10^9 motile sperm would be most practical for pig production with limited genetic resources. Fertility was also influenced by boar, interval from insemination to ovulation, and gilt ovarian abnormalities.

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1. Introduction

Due to reduced fertility in comparison to liquid extended semen, frozen-thawed boar sperm is used in less than 1% of all inseminations (Johnson et al., 2000). Despite its limited use in commercial production, frozen boar sperm has potential for application if improvements can be achieved in post-thaw sperm quality and AI fertility. Frozen boar semen is valued for maintaining swine genetics in the cryopreserved state to allow for preservation and regeneration of valued genetic lines, for providing for an emergency sperm supply, and for facilitating opportunities for global gene distribution (Almlid and Hofmo, 1996; Johnson et al., 2000; Eriksson et al., 2002; Roca et al., 2006; Bailey et al., 2008).

Extensive research has been conducted on methods to improve post-thaw fertility of frozen boar sperm. One major limitation is that fertility following freezing is influenced by boar and ejaculate and involves variability in plasma membrane integrity (Waterhouse et al., 2006). To preserve membrane integrity and sperm viability, several freezing methods have proven successful (Larsson, 1978; Bwanga, 1991; Johnson et al., 2000; Grobfeld et al., 2008). Of these, computerized, controlled-rate freezing machines (Johnson et al., 2000; Thurston et al., 2003) allow for optimal freezing rate control for maintaining sperm membrane integrity (Fiser et al., 1993; Hernandez et al., 2007b). In addition, sperm freezing in straws and bags allows for more uniform freezing and thawing with the increased surface area when compared to pellets (Pursel and Johnson, 1976; Bwanga et al., 1990; Eriksson and Rodriguez-Martinez, 2000a,b). Improvements in freezing media, such as addition of cholesterol (Bailey et al., 2008), seminal plasma (Hernandez et al., 2007a; Okazaki et al., 2009b) or antioxidants (Grobfeld et al., 2008) have been reported to have potential for improving post-thaw sperm motility and acrosome integrity.

Many early studies with frozen boar sperm resulted in reduced post-thaw quality, and fertility following insemination. The decreased sperm fertility may have resulted as a consequence of method of freezing while reduced AI fertility could be attributed to variation in number of inseminations, number of sperm used, and less than optimal AI timing. In studies, the insemination doses tested were based on the number of motile or total sperm cells per dose with estimates for motility averaging ~30% (Einarsson et al., 1973; Larsson and Einarsson, 1976). In a review of earlier research, farrowing rates remained at ~55% and litter sizes at ~8 pigs/litter (Johnson, 1985). More recent studies have used single or double inseminations with 5 to 6×10^9 total sperm with post-thaw motility reported at ~40–60% (Roca et al., 2003; Okazaki et al., 2009a). Fertility following AI in more recent trials has yielded pregnancy rates >60% with litter sizes >9 pigs per litter (Hofmo and Grevel, 2000; Eriksson et al., 2002). New technologies, such as intrauterine and deep uterine AI, may allow for increased fertility when using reduced numbers of sperm (Roca et al., 2003; Rozeboom et al., 2004). Improved AI timing is also essential because frozen boar sperm has a reduced fertile lifespan (Einarsson and Viring, 1973; Waberski et al., 1994) and must be deposited within 4 h before ovulation to result in fertilization rates similar to those using liquid semen (Waberski et al., 1994).

We hypothesized that improved pregnancy rates and litter sizes would result from use of increased numbers of motile, frozen-thawed sperm when using multiple instead of single timed inseminations in order to compensate for variation in the time of ovulation and reduced fertile lifespan of frozen sperm. The objectives of this study were to test the impact of numbers of motile, frozen-thawed boar sperm in either a single or double AI, in relation to interval from last estrous synchronization treatment to estrus, time of ovulation, and other sources of variation that included boar, ovulation rate, quality of insemination, and other uncontrolled causes of variation.

2. Materials and methods

2.1. Animals, induction and estrous synchrony, ovulation and insemination determination

The experiment was performed at the University of Illinois swine research farm in six sequential replicates from December 2007 to June 2008. All procedures utilized in this experiment were approved by the University of Illinois Institutional Animal Care and Use Committee. Terminal line gilts ($n = 123$, 337 × C22, PIC North America, Hendersonville, TN) were moved from a finishing barn into crates in a gestation building. All gilts were given PG600 (400 IU eCG and 200 IU hCG, Intervet/Schering-Plough, Kenilworth, NJ) for initial induction of estrus. The gilts selected for this experiment ranged from 165 to 198 days of age and weighed between 95 and 139 kg at the time of induction. Estrus detection was performed daily using the back-pressure test while providing fenceline contact with a mature boar. Ten days following treatment with PG600, regardless of estrus expression, stage of estrous cycle in gilts was synchronized using a synthetic progestagen, MATRIX (Altrenogest 2.2 mg/mL, Intervet/Schering-Plough, Kenilworth, NJ, USA) for 14 days as a top-dress on a standard sow gestation diet. After 14 days of MATRIX feeding, in replicates 1–3, gilts were checked for estrus once daily. In replicates 4–6, PG600 was given to all gilts 24 h following LMF to reduce variation in timing of estrus and to aid in labor scheduling and timing for AI. Beginning on the third day following LMF, estrus detection was performed at 12 h intervals. Transrectal real-time ultrasonography (Aloka 500 V, Tokyo, Japan) was initiated 12 h following onset of estrus and continued at 12 h intervals to observe ovaries and estimate time of ovulation (Knox et al., 2002). The total number of large antral follicles >5.0 mm was counted and the images recorded in electronic format on DVD for playback confirmation of follicle size and number. Overall, 111 animals expressed estrus after LMF and were assigned to treatment. At onset of estrus, gilts were randomly assigned in a 3 × 2 factorial treatment design to receive 1×10^9 motile FTS ($n = 19$), 2×10^9 motile FTS ($n = 19$), 4×10^9 motile FTS ($n = 19$) in a single AI at 32 h after onset of estrus, or 1×10^9 motile FTS ($n = 18$), 2×10^9 motile FTS ($n = 17$), or 4×10^9 motile FTS ($n = 19$) in each of the two AI at 24 and 32 h following onset of estrus. Gilts were randomly assigned to

each treatment in order of estrus detection so that all treatments would receive sperm from the same boar. The order for boar use was random, but we selected each boar to be represented across each treatment before the process was repeated for next boar. The daily experimental procedures involved semen thawing and dose preparation (0500 and 1700 h), ultrasonography (0800 and 2000 h), followed by estrous detection (0900 and 2100 h).

2.2. Semen collection, evaluation, and freezing

Terminal line (L220), excellent health, crossbred boars (PIC, $n = 6$) with a previous history of offspring production were selected for use in this study. A total of 3 to 7 ejaculates were collected for each boar and processed for freezing. Semen was collected at a commercial PIC stud, diluted 1:4 (v/v) with 37 °C Androhep Plus (Minitube of America, Verona, WI), cooled to 15 °C and shipped overnight to the USDA-ARS National Center for Genetic Resources Preservation, National Animal Germplasm Program in Fort Collins, CO, USA. The samples were then centrifuged for 10 min at $800 \times g$ and the supernatant aspirated and the resulting pellet evaluated for sperm concentration using a spectrophotometer (Purdy, 2008). Motility of the ejaculate was determined by Computer Assisted Sperm Analysis (CASA, Hamilton Thorne Research, Beverly MA, USA) as previously described (Purdy, 2008).

The ejaculates used in this experiment were frozen using the method of Pursel and Johnson (1975) as modified by Purdy (2008). Briefly, following centrifugation, sperm pellets were re-suspended in BF5 cooling extender (CE, 52 mM TES, 16.5 mM Tris[hydroxymethyl] aminomethane, 178 mM glucose, 20% egg yolk; 325 mOsm) to 750×10^6 sperm/mL. The sperm were then equilibrated at 5 °C for 2.5 h and diluted to 500×10^6 sperm/mL by drop-wise addition of the BF5 freezing extender (91.5% CE, 6% glycerol, 2.5% Equex Paste, Minitube of America; 1450 mOsm (Pursel and Johnson, 1975). The sperm was placed into 0.5 mL CBS straws (IMV Corporation, Minneapolis, MN, USA) and frozen using a programmable freezer (Minidigicool UJ400, IMV USA) with the following freeze curve: 5 to −8 °C at 20°/min; −8 to −120 °C at 69 °C/min; −120 to −140 °C at 20 °C/min (Purdy, 2008). Following the freezing cycle the straws were placed directly into liquid nitrogen for storage.

Following ejaculate freezing, the numbers of motile sperm/straw were estimated by thawing a random straw and evaluating motility using CASA. Thawed sperm were diluted 1:5 (v/v) in BTS and warmed to 37 °C and evaluated after 10 min. The CASA used the following variables which were preset by the manufacturer: 30 frames acquired, frame rate of 60 Hz, minimum contrast of 55, minimum cell size of 5 pixels, VAP cutoff of 20 μm, progressive minimum VAP cutoff of 45 μm/s, VSL cutoff of 5 μm/s, static head size of 0.53–4.45, and magnification of 1.89. A minimum of seven fields and 1000 sperm were observed for motility analysis. The percentage of motile sperm within each straw was used to determine the number of straws needed to create doses with the proper number of motile sperm. Each dose of FTS was comprised of each of 3–7 ejaculations from a single boar. Frozen sperm to be used for insemination was sent to the University of Illinois in liquid nitrogen dry vapor containers.

2.3. Sperm thawing and insemination

To prepare each insemination dose, straws of frozen boar sperm were placed in a 50 °C waterbath and agitated for 20 s for thawing. The thawed sperm were diluted with 80 mL of Androhep CryoGuard Thaw extender (Minitube of America, Verona, WI) in a 26 °C waterbath. Within 30 min of thawing, intra-cervical insemination was performed using standard spirette or foam tipped AI catheters (Minitube of America, Verona, WI) in the presence of a boar. Because all animals in this experiment received an insemination at 32 h following first expression of estrus, this AI was scored for fluid loss during AI. Insemination scoring was based on categorical 1–3 scale, with a score of 1 having excessive volume loss during AI, 2 having moderate loss, and 3 having no volume loss during insemination.

2.4. Pregnancy classification and reproductive tract processing

Gilts were sacrificed between days 28 and 34 of gestation and reproductive tracts collected. The tracts were identified and processed for pregnancy status (0 fetus = not pregnant, or 1 or more fetus = pregnant), number of normal fetuses, number of degenerative fetuses (those with abnormal appearance), number of corpora lutea (CL), and presence of any abnormal structures associated with the ovaries or oviducts. Abnormalities were identified as follicular or luteal cysts (>12.9 mm) or fluid-filled oviducts.

2.5. Statistical evaluation

Data were evaluated in SAS (SAS Institute, Inc., Cary, NC) using a mixed model ANOVA (PROC MIXED) for all continuous response variables and PROC GENMOD for binary response variables. The continuous response variables included age and weight of gilts, number of normal fetuses, quality of insemination, number of degenerative fetuses, and embryonic survival (total number of fetuses divided by number of CL at slaughter). The binary response variables included: pregnancy at slaughter. The MIXED and GENMOD models included the main effects of dose, number of inseminations, the interaction of dose × number of inseminations, boar, AI score, and replicate. Additional continuous variables such as number of large follicles at estrus and number of CL at slaughter were included as covariates to test for linear, quadratic and cubic effects. To test for the effects of interval from insemination to ovulation and an abnormal ovary at slaughter on pregnancy rate and number of normal fetuses, one-way ANOVA procedures were performed using the MIXED and GENMOD procedures, respectively.

To examine the effects of the procedures used for induction and synchronization and use of PG600 following LMF, one-way ANOVA was performed using PROC MIXED with replicate group (1–3 or 4–6) and expression of estrus (Yes or No) following initial PG600 treatment as class variables in the model. The response variables included the interval

from LMF to estrus, duration of estrus, interval from insemination to ovulation, numbers of follicles and CL, pregnancy rate and number of fetuses. All means comparisons were performed using the LSMEANS statement in SAS and differences determined using the PDIFF option. In addition, Pearson product moment–correlation analyses were performed using the PROC CORR function in SAS to determine the simple correlation relationship between the number of follicles >5.0 mm counted using ultrasound with the final number of CL counted at slaughter.

3. Results

3.1. Post-thaw sperm motility

A total of 26 ejaculates were frozen from the six boars for use in this experiment with post-thaw motility averaging 38%. Each insemination dose included all ejaculates from the same boar. The post-thaw motility for each boar averaged 38 ± 2.9% for boar A (three ejaculates), 36 ± 4.0% for boar B (five ejaculates), 46 ± 3.5% for boar C (four ejaculates), 32 ± 2.8% for boar D (seven ejaculates), 33 ± 6.4% for boar E (four ejaculates), and 37 ± 3.8% for boar F (three ejaculates). Each 0.5 mL straw contained ~94×10^6 motile sperm cells.

3.2. Response to estrous induction and synchronization

The initial pubertal response to the estrus induction treatment with PG600 was 66% with an interval of 100 ± 2 h (mean ± SE) from PG600 to estrus, with estrus lasting 42 ± 2 h. Expression of estrus following the pubertal induction with PG600 had no effect ($P > 0.20$) on expression of estrus following LMF, or on final pregnancy rate. Use of PG600 following LMF reduced the interval from LMF to estrus ($P < 0.0001$) in replicates 4–6 (130 ± 3 h) compared to replicates 1–3 (148 ± 3 h). Use of PG600 following LMF also reduced the duration of estrus ($P < 0.0005$) in replicates 4–6 (42 ± 3 h) compared to replicates 1–3 (52 ± 3 h). However, the interval from onset of estrus to ovulation was not influenced by use of PG600 ($P > 0.20$), and averaged 33 ± 1 h in replicates 1–3, and 34 ± 1 h replicates 4–6. PG600 increased ($P < 0.0001$) the numbers of large follicles at estrus in replicates 4–6 compared to replicates 1–3 (22 compared to 16), but there was no effect of replicate on number of CL at slaughter (17). There were no effects of use of PG600 following LMF on pregnancy at slaughter or on number of fetuses.

The number of large follicles (>5 mm) counted during ultrasound and then validated by counting from playback of the digital recording on a digital imaging system was correlated ($r = 0.90$, $P < 0.0001$) and averaged 19.0 ± 0.7 follicles, but were unrelated to the number of CL at slaughter (17.0 ± 0.3). The AI score at 32 h following estrus did not differ among doses or number of inseminations ($P > 0.20$) and averaged 2.7 ± 0.1 (3.0 = no volume loss), with 73% of inseminations having an AI score = 3, while 24% had an AI score = 2, and 3% had an AI score = 1. Across all replicates at the time of slaughter, 7.2% of gilts had ovaries that were classified as abnormal, with six gilts having cystic structures and two gilts showing fluid-filled oviducts.

3.3. Impact of dose and AI number on pregnancy rate and litter size

The average age (168.8 ± 1.2 days) and weight (114.5 ± 1.0 kg) of gilts assigned to each treatment based on estrus were not different among treatments (>0.20).

3.3.1. Pregnancy

Pregnancy rate at slaughter averaged 73.0 ± 4.2%. There was no interaction of dose and number of AI and no effect of dose or number of inseminations (Table 1). There was no effect of boar (Table 2), but interval from insemination to ovulation influenced pregnancy rate ($P < 0.05$, Table 3). Increased pregnancy rates were observed when insemination occurred at 4 h before ovulation or up to 8 h after ovulation, compared to earlier or later intervals. While most additional sources of variation had no effect on pregnancy, the presence of an abnormal ovary was associated with only a 12.5% pregnancy rate ($P < 0.0001$).

3.3.2. Litter size

There was no significant interaction of dose and number of inseminations and no effect of dose on numbers of normal fetuses (Table 1). However, number of inseminations tended to influence numbers of normal fetuses ($P = 0.14$) with double inseminations resulting in greater numbers of normal fetuses (11.5 ± 0.6) compared to single inseminations (10.2 ± 0.6). Boar influenced numbers of normal fetuses ($P < 0.01$) with boar A having the smallest and boar D the largest litter size (Table 2). Number of normal fetuses increased as insemination occurred closer to ovulation ($P < 0.001$, Table 3). Additional sources of variation had no effect on normal fetus numbers. Number of degenerat-

Table 1
Least squares means for pregnancy and litter data in response to treatment with dose of motile, frozen-thawed sperm (1 to 4×10^9) and number of inseminations (single or double). Data were collected from mature gilts between 28 and 34 days of gestation.

Motile sperm ($\times 10^9$)	Number of AI[a]	Pregnancy at slaughter (%)[b]	Normal fetuses	Degenerative fetuses	Embryonic survival (%)
1	1	76.4 (14/19)	9.5 ± 1.1	0.2 ± 0.4	61.5 ± 5.8
1	2	70.4 (13/19)	11.3 ± 1.2	0.0 ± 0.4	65.6 ± 6.1
2	1	71.1 (13/19)	10.4 ± 1.2	0.4 ± 0.4	67.3 ± 6.0
2	2	79.2 (14/18)	12.6 ± 1.1	0.1 ± 0.4	71.0 ± 5.7
4	1	66.1 (12/17)	10.6 ± 1.2	0.7 ± 0.4	59.2 ± 6.5
4	2	87.3 (15/19)	10.7 ± 1.1	1.0 ± 0.4	69.5 ± 5.8

[a] AI occurring at 32 h after detection of estrus for single AI and at 24 and 32 h for double AI.
[b] Numbers in parentheses are proportions of gilts.

Table 2
Least squares means for pregnancy and litter data in response to the main effect of boar in GENMOD and MIXED models following insemination with 1 to 4×10^9 motile, frozen boar sperm in a single or double AI. Data were collected from mature gilts between 28 and 34 days of gestation.

Boar	Pregnancy at slaughter (%)	Normal fetuses	Degenerative fetuses	Embryonic survival (%)
A	78.6 (15/20)	7.2 ± 1.1^{z}	0.4 ± 0.4	42.9 ± 6.1^{z}
B	77.7 (14/18)	9.8 ± 1.1^{x}	0.1 ± 0.4	74.1 ± 6.1^{xy}
C	73.1 (13/19)	10.8 ± 1.1^{xy}	0.1 ± 0.4	65.1 ± 6.2^{xy}
D	71.4 (13/18)	13.8 ± 1.2^{y}	0.5 ± 0.4	75.8 ± 6.6^{y}
E	83.9 (15/18)	12.6 ± 1.1^{y}	0.4 ± 0.4	74.9 ± 6.0^{xy}
F	65.7 (11/18)	10.9 ± 1.3^{xy}	1.1 ± 0.4	61.3 ± 6.8^{x}

[xyz]Different superscripts within a column indicate differences ($P < 0.05$). Numbers in parentheses are proportions of gilts.

Table 3
Least squares means resulting from GENMOD and MIXED models for pregnancy and litter data in response to estrus to ovulation interval following insemination with 1 to 4×10^9 frozen boar sperm in a single or double AI. Data were collected from mature gilts between 28 and 34 days of gestation.

Estrus to ovulation (h)[a]	Interval from last AI at 32 h to ovulation[b]	Pregnancy at slaughter (%)	Normal fetuses	Degenerative fetuses	Embryonic survival (%)
12	+20	50.0 (2/4)^{xz}	15.0 ± 3.0^{y}	0.0 ± 1.0	75.5 ± 16.8^{y}
24	+8	80.0 (27/35)^{y}	12.1 ± 0.8^{xy}	0.4 ± 0.4	76.0 ± 4.7^{y}
36	−4	88.1 (37/43)^{y}	11.1 ± 0.7^{xy}	0.2 ± 0.3	64.0 ± 4.1^{y}
48	−16	57.8 (11/19)^{x}	6.0 ± 1.3^{z}	0.1 ± 0.5	44.3 ± 7.2^{x}
60	−28	0.0 (0/1)^{z}	–	–	–

Numbers within parentheses are proportions of gilts.
[x,y,z]Different superscripts within column indicate differences ($P < 0.05$).
– No estimate.
[a] Estrous detection and transrectal ultrasonography were conducted at 12 h intervals.
[b] Use of ultrasonography allowed for observation of ovulation in 101 of 111 gilts.

ing fetuses/litter averaged 0.4 ± 0.2 and was not influenced by dose and number of inseminations. Embryonic survival ($65.5 \pm 3.0\%$) was not influenced by dose and number of inseminations or other variables but was affected by boar ($P < 0.005$, Table 2) and interval from insemination to ovulation ($P < 0.005$, Table 3).

4. Discussion

The overall pregnancy rates (73%) and number of normal fetuses (10.8) observed in the present study were somewhat higher than expected. It is not clear whether the gilts in this study would have actually maintained pregnancy to term and whether the normal fetus numbers would survive to be liveborn pigs, but assessments for fetal and ovarian measures for normality indicated there would be few limitations. The aim of this study was to demonstrate that increasing the dose of motile, frozen-thawed sperm with double AI would result in acceptable pregnancy rates and litter sizes by compensating for reduced sperm quality and variation in time of ovulation. There was no indication that dose of frozen sperm interacted with numbers of inseminations to compensate for poor sperm fertility or variation in time of ovulation. However, double insemination tended to improve litter size compared to single insemination and as expected, interval from insemination to ovulation affected both pregnancy rate and litter size.

Collectively it would appear that the technology for frozen boar sperm use can be reliable for producing pregnancy rates at ~70% and litter sizes of ~10 pigs. Most studies testing fertility of frozen-thawed boar sperm utilize frozen sperm numbers equivalent to 2.5 to 3.0×10^9 motile cells or 5 to 6×10^9 total sperm with similar fertility outcomes shown in the present trial (Johnson, 1985; Roca et al., 2006). In the present study, dose had no effect on pregnancy or litter size. From a practical standpoint, using the least amount of sperm would have the greatest advantages. Our test with 1×10^9 motile sperm in single or double AI resulted in pregnancy rates ≥70% and numbers of normal fetuses between 9.5 and 11.3. Most of the treatments resulted in acceptable fertility for use of frozen boar sperm. Results in the present study are similar to others where greater numbers of sperm were used and with comparable numbers of gilts (Eriksson and Rodriguez-Martinez, 2000a), and also where greater numbers of sperm and even larger numbers of sows were used (Eriksson et al., 2002). The effect of sperm dose has been tested for both liquid and frozen boar sperm. In some frozen boar sperm studies, altering dose had variable effects for pregnancy and litter size responses (Einarsson et al., 1973; Larsson et al., 1977; Johnson, 1985). Yet in certain studies with liquid semen (Watson and Behan, 2002) or with small dose, or deep uterine insemination (DUI) of frozen sperm, the effects of dose were quite clear (Bolarín et al., 2006). The reasons for the discrepancies among studies may reflect differences in any of the following variables: actual sperm dose (motile or total sperm used), number of inseminations, use of single sire or pooled semen, boar fertility, AI timing, and the reproductive maturity and management of the animal used.

Number of inseminations did not affect final pregnancy rate, but did tend to influence number of normal fetuses. It was surprising that there were limited effects of number of inseminations, since a double AI system of frozen-thawed boar sperm had previously shown greater non-return rates and increased litter sizes in studies with fewer (Einarsson et al., 1973) and even more animals tested (Reed, 1985). In contrast to the present study, it is possible that the ear-

lier methods of cryopreservation allowed for compensable fertility in response to number of inseminations. However, although not significant in the present study, at the greater doses, pregnancy rates were greater, and this is similar to farrowing rate increases that have been noted with double AI compared to single AI in sows (Almlid et al., 1987). Yet only a few studies have been conducted that have tested dose of frozen sperm with number of inseminations (Einarsson et al., 1973; Bathgate et al., 2008). In these studies, regardless of the AI method used (cervical or DUI), results indicate that more inseminations at greater doses yield greater fertility.

In the present experiment there was no clear choice for optimizing final pregnancy establishment or litter size. The greatest pregnancy rates were observed with 2 and 4×10^9 motile sperm with double AI, while the largest litter sizes occurred with 1 and 2×10^9 motile sperm in a double AI. To examine the fertility and sperm use efficiency, fertility indices were created for total number of normal pigs produced/treatment (final pregnancy rate ($\times 100$) $\times$ number of normal fetuses) and numbers of motile sperm/piglet produced (total number of motile sperm used per treatment/total number of normal pigs produced per treatment). The fertility index revealed that double AI of 1, 2, and 4×10^9 sperm produced more pigs (796, 998, and 934 pigs) than single AI (726, 739, and 701 pigs, respectively). The index for number of sperm used per pig produced indicated that single insemination with 1×10^9 motile sperm was most efficient (0.14×10^9 sperm/pig). However, the greatest number of pigs was produced when using 2×10^9 motile sperm in a double insemination. If equal weight is given to both numbers of pigs produced and the numbers of sperm used for each pig produced, the double insemination at 1 and 2×10^9 motile cells allowed for the most pigs produced with least amount of sperm used per pig (0.25 and 0.40×10^9 sperm/pig, respectively). For production purposes a double insemination using 2×10^9 motile sperm would produce the greatest number of pigs consistently when using the least amount of frozen sperm. When access to valuable genetics is limited by increased demand, a double insemination using 1×10^9 motile sperm would allow for acceptable pig production with minimal sperm use.

Compensating for variation in the time of ovulation was a major objective of the present study and the reason why double insemination was included. Double insemination is standard practice for commercial pig production when utilizing liquid semen (Flowers and Esbenshade, 1993; Kemp et al., 1996). The timing system in the present study for the single and double AI following detection of estrus has been used in experiments with frozen sperm in gilts and sows with single (Johnson et al., 1982; Aalbers et al., 1985) and double inseminations (Rodriguez-Martinez et al., 1996; Eriksson and Rodriguez-Martinez, 2000a; Eriksson et al., 2002). These published trials with gilts showed pregnancy rates of ~60% (Rodriguez-Martinez et al., 1996; Eriksson and Rodriguez-Martinez, 2000a), while the farrowing rates for the sow trials averaged ≥70% (Eriksson et al., 2002). The AI timing system in the present study was designed with a predicted estrus to ovulation average of 36 h (Almeida et al., 2000; Bortolozzo et al., 2005; Horsley et al., 2005). In the present study, time of ovulation averaged 33 h. We observed that 34% of gilts had ovulations by 24 h, 42% by 36 h, and 19% by 48 h. As a result, the majority of inseminations occurred close to ovulation, which is important since frozen swine sperm has a shortened fertile lifespan *in utero* (Waberski et al., 1994). In the present study, interval from estrus to ovulation affected pregnancy rate, litter size, and embryonic survival similar to frozen sperm experiments using DUI (Bolarín et al., 2006). These fertility measures were greatest when gilts ovulated by 24 and 36 h. For gilts that ovulated by 48 h, pregnancy rate dropped to ~58% and litter size declined to 6.0 fetuses. Collectively, all studies show the importance of frozen sperm AI occurring close to the time of ovulation (Waberski et al., 1994; Wongtawan et al., 2006). Perhaps in the future, synchronization of ovulation could be used to prevent delayed ovulation when using frozen sperm (Okazaki et al., 2009a).

Although not novel, it is still important to account for the fact that in this and other studies, boar affects the number of normal fetuses, and embryonic survival (Almlid et al., 1987; Eriksson and Rodriguez-Martinez, 2000a). Because gilts in the present study were inseminated based on total numbers of motile sperm, boar differences in this measure were not a factor in gilt fertility. However, variance in post-thaw motility was evident among boars and ejaculates. Post-thaw motility averaged 38% and was lower than the 50% motility reported by (Eriksson et al., 2002; Roca et al., 2003) while farrowing rates and litter sizes were similar. However, poor motility does not always translate to reduced fertility for liquid or frozen boar sperm (Flowers, 1997; Eriksson et al., 2002). It should be noted that in some experiments, boars are selected based on post-thaw sperm survival for use in frozen sperm fertility tests (Hernandez et al., 2007a; Okazaki et al., 2009b). However, in the present study, boars were selected on previous history for commercial litter production with liquid semen. As a result, we did observe boar fertility differences similar to that reported by (Flowers, 2002).

While the present experiment focused on the primary fertility measures of pregnancy rate and number of normal fetuses in early gestation, the timing of animal sacrifice allowed us to obtain data on numbers of degenerative fetuses and embryonic survival. Both factors provided us with an indication of the overall health of the litter. Additional variables were also examined for their effects on fertility when using frozen boar sperm. Insemination technique had no effect on fertility since the AI score was very good. This was important to test since the volume of the inseminate has been reported to affect sperm transport, the sperm reservoir, and fertilization (Baker et al., 1968). Number of follicles at estrus and number of CL also did not influence litter size or measures of litter health. However, previous studies have suggested that ovulation rate could influence embryonic survival, pregnancy, and litter size during early gestation (Town et al., 2005; Foxcroft et al., 2007). Because follicle numbers and ovulation rate were within normal limits, it is not surprising that we did not observe any effect on fertility.

In the present experiment prepubertal gilts induced with PG600 were used, with subsequent progestagen synchronization to measure fertility from use of frozen boar

sperm. Gilt weight, age and initial estrus had no effect on estrus synchronization or fertility outcomes. Despite an initial estrus induction response <70%, subsequent synchronization with Matrix resulted in an estrous synchrony response of >90% similar to other reports (Estienne et al., 2001). As a result, it is possible that insemination may have occurred at pubertal estrus for ~30% of gilts. Assessment of our synchronized gilt model for fertility indicated normal measures for duration of estrus, interval from estrus to ovulation, numbers of large follicles at estrus, and numbers of CL (Bracken et al., 2003; Horsley et al., 2005; Breen et al., 2006). The effect of PG600 on reducing the interval to estrus without influencing interval from estrus to ovulation has been observed in weaned sows (Knox et al., 2002) and suggests that the use of PG600 changes the time of ovulation from 63% of the way through estrus (replicates 1–3) to 78% (replicates 4–6). Numbers of CL can be an important component to consider when accounting for differences in fertility responses. To account for possible fertility effects, we counted large follicles at estrus and correlated these to digital recordings. Others have also examined this and correlated ultrasonographic measures with use of laparoscopy (Bolarin et al., 2009). However, it is of interest to note that follicle counts were not correlated with the number of CL at slaughter as has been reported previously (Soede et al., 1992). It is possible that this discrepancy might be explained by use of PG600 on follicle development or by the occurrence of follicle heterogeneity and the differential response to the LH surge for ovulation (Hunter and Wiesak, 1990; Knox, 2005). Finally, in the present study, it was noted that 7% of gilts had cystic ovaries or fluid-filled oviducts. In either case, this had a negative impact on pregnancy rate and number of normal fetuses. These occurrences were unrelated to use of PG600 and these problems have been observed in mature gilts and sows (Heinonen et al., 1998) with associated reduction in fertility (Waberski et al., 2000; Castagna et al., 2004).

5. Conclusion

There was no interaction of dose and number of inseminations when using frozen boar sperm on fertility. The results of this study indicate that double insemination with 1 to 2×10^9 motile, frozen-thawed boar sperm could be used to achieve acceptable pregnancy rates and litter sizes with the most efficient use of sperm. Use of single AI was less efficient for pig production while use of 4×10^9 motile sperm in a double AI was less efficient for use of sperm. Variation in interval from AI to ovulation and boar fertility accounted for significant fertility effects. Methods to control ovulation time and maximize boar fertility would aid this technology. The induction and estrous synchronization procedure for prepubertal gilts proved to be an effective method for testing frozen sperm fertility.

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